



[DESCRIPTION]

PROCESS FOR PRODUCING PEPTIDES USING A HELPER PEPTIDE

~~Technical Field~~

5       ~~The present invention relates to a process for producing peptides using genetic engineering technology.~~

[Background Art] BACKGROUND OF THE INVENTION

10       Many [biologically active] biologically-active peptides have been produced by genetic engineering technology using microorganisms, animal cells, and the like as hosts. [As processes] Processes for producing a peptide of interest[, there are known] include the "direct expression method." ~~so-called direct~~  
15       ~~expression method such as a process for~~ Direct expression methods include: extracellularly secreting a peptide, [or a process for] expressing a peptide from the [N-terminal] N-terminus in the cell[,; or a process for] expressing a fusion protein in which a  
20       protective peptide has been added to the [N-terminal or the C-terminal] N-terminus or the C-terminus of the peptide[, or the like]. Processes have been used wherein a peptide of interest is expressed either inside or outside of the cell[, the] The expression  
25       product is then subjected to chemical or enzymatic cleavage or other modification to produce the peptide of interest[, which] The peptide of interest is then purified in a purification process to yield the peptide.

30       For the production of low molecular weight peptides, the foregoing fusion protein expression process [mentioned above] is generally used in order to avoid degradation of the protein by intracellular proteolytic enzymes. In this case, processes have  
35       been used wherein after intracellular expression of a

fusion protein having a cleavage site region added thereto, in between the peptide of interest and the protective peptide (said cleavage site region being designed so as to permit cleavage by a chemical or enzymatic reaction), the peptide of interest is cleaved from the fusion protein by a chemical or enzymatic means[,] and the peptide is isolated and purified by a precipitation or a chromatographic process.

Furthermore, when the protein of interest is a protein of which a [C-terminal] C-terminus has been amidated, such as calcitonin, a peptide having a glycine residue added to the [C-terminal] C-terminus end of said peptide is expressed as a part of a fusion protein, the glycine-extended peptide of interest is cleaved from the fusion protein by a proteolytic enzyme, an amidating enzyme which is a modification enzyme is allowed to act on the peptide to produce an amidated peptide, and the amidated peptide of interest is obtained through purification processes.

However, when the production of peptides on an industrial scale is desired, various problems may arise, such as: [to] the solubility and gelling of the peptide of interest in various conditions for cleaving and modification reactions, the concentration of [a] sample to be loaded [to] onto the column in the chromatographic process, the elution condition from the column, stability after elution, and the like. Most of the problems are caused by various physicochemical properties of the peptide of interest.

~~As the peptide of interest, for example, there can be mentioned human glucagon-like peptide-1 (referred to hereinafter as GLP-1, Human glucagon-like peptide-1 ("GLP-1") (Bell GI et al., Nature, Vol. 304, pp. 368-371, 1983) and derivatives of GLP-1 (referred~~

to hereinafter as GLP-1 derivatives) ("GLP-1 Derivatives") having an insulintropic activity are examples of peptides of interest. GLP-1 is a peptide consisting of 37 amino acid residues derived from preproglucagon. Through processing of preproglucagon, [GLP-1(7-37)] "GLP-1(7-37)" (in which 6 amino acids at the [N-terminal] N-terminus of GLP-1 are deleted) and [GLP-1(7-36)NH<sub>2</sub>] "GLP-1(7-36)NH<sub>2</sub>" (in which the [C-terminal] C-terminus of GLP-1(7-36) has been modified to an amide form) have been biosynthesized (Mojsov, S. et al., J. Clin. Invest. Vol. 79, pp. 616-619, 1987). Since these peptide hormones [have an activity of promoting] promote the secretion of insulin by acting on the beta cells in the pancreas, it has recently been proposed that they may be therapeutic agent candidates for diabetics ~~the possibility as candidates of a therapeutic agent for diabetics has been proposed~~ based on their pharmacological effects [in recent years] (Gutniak M. K., et al., New England Medicine, Vol. 326, pp. 1316-1322, 1992; Nathan D. M., et al., Diabetes Care, Vol. 15, pp. 270-275, 1992).

~~As a process for producing the above~~ Such a peptide, a case may be contemplated in which the peptide is may be produced by a fusion protein expression method using *E. coli* [etc.] or the like as a host in the conventional method mentioned above. In the case of GLP-1(7-37), for example, it is expressed as a fusion protein in which a protective peptide is added to the [N-terminal or C-terminal end] N-terminus or C-terminus of GLP-1(7-37) through a cleavage site region for excising out GLP-1(7-37) from the fusion protein either chemically or enzymatically. [, and then] GLP-1(7-37) can then be excised [out] from the fusion protein, either chemically or enzymatically, to produce GLP-1(7-37). [And,] GLP-1(7-36)NH<sub>2</sub> can be

produced by adding a [step of] modification reaction  
step to the [above] foregoing process. Thus, GLP-1(7-  
37) is expressed as a fusion protein as mentioned  
above as a substrate for an amidation enzyme for the  
amidation modification reaction wherein GLP-1(7-37)  
can be considered as an intermediate peptide for the  
production of GLP-1(7-36)NH<sub>2</sub>. GLP-1(7-37) is excised  
from the fusion protein, either chemically or  
enzymatically, and then the GLP-1(7-37) thus obtained  
is subjected to an amidation modification reaction  
using an amidation enzyme to produce the desired GLP-  
1(7-36)NH<sub>2</sub>.

~~However, even when the above mentioned processes~~  
~~are used to produce a peptide of interest, a GLP-1~~  
~~derivative having an insulintropic activity,~~  
~~undesirable problems on production processes arise.~~  
arise when producing a desired GLP-1 derivative having  
insulintropic activity using the foregoing processes.  
Therefore, there is a strong, unmet need for  
production processes that permit inexpensive supply on  
an industrial level ~~have not been established yet, and~~  
~~thus there is a strong need for the establishment of~~  
~~such methods.~~

For example, [as a process] one method for  
producing GLP-1(7-37) as a peptide of interest  
utilizes a fusion protein comprising a protective  
peptide and GLP-1(7-37) [is] expressed [in] using an  
conventional process, as mentioned above. [, and] GLP-  
1(7-37) can then be produced by excising it directly  
from said protein. [Thus, the purification]  
Purification steps of the production process can use  
(1) enzymatic excision [step] of GLP-1(7-37) from the  
fusion protein and (2) a [chromatographic step]  
chromatography. However, since GLP-1(7-37) can easily  
gel or aggregate during purification, production

problems ~~on production processes that are derived from~~  
related to physicochemical properties, such as  
markedly reduced recovery and incapability of resin  
regeneration, may sometimes arise. Once it has been  
5 gelled, though it can be solubilized at a pH of 10 or  
higher and can be purified, problems such as  
undesirable modifications and conformational changes  
may appear (Senderoff RI, et al., J. Pharm. Sci., Vol.  
87, pp. 183-189, 1998).

10 Furthermore, when the peptide of interest is an  
amidated peptide GLP-1(7-36)NH<sub>2</sub>, problems may arise [as  
to] resulting from the amidation reaction. Thus,  
optimal pH of enzymatic amidation reactions is  
approximately from weak acid to neutral, whereas the  
15 theoretical isoelectric points ("pI") of GLP-1(7-37)  
and GLP-1(7-36)NH<sub>2</sub> are [pI =] 5.5 and [pI =] 7.5,  
respectively. Therefore, when an enzymatic conversion  
to GLP-1(7-37) is carried out at the optimal pH of an  
amidation enzyme that is near the isoelectric point of  
20 the substrate GLP-1(7-37), it is believed that GLP-  
1(7-37) tends to form an isoelectric precipitation.

Furthermore, since GLP-1(7-36)NH<sub>2</sub> that was formed  
by the precipitated GLP-1(7-37) also coprecipitates,  
enzyme reactions may not fully proceed, causing  
25 production problems [in production processes].  
Furthermore, since GLP-1(7-36) is also a peptide that  
tends to aggregate in handling (during the column  
step) and [to] gel in the column, purification  
problems [as to purification] may also arise. [Thus,  
30 it] It is expected that GLP-1 derivatives also have  
the above-mentioned problems, due to their ~~in~~  
~~production processes, that are derived from~~  
physicochemical properties.

When producing [In the production of] GLP-1(7-  
35 37), GLP-1(7-36)NH<sub>2</sub>, and GLP-1 derivatives on an

industrial scale, the above-mentioned problems can,  
due to the physicochemical properties of the peptide  
of interest itself, ~~cause problems on industrial~~  
~~production derived from the physicochemical properties~~  
5 ~~of the peptide of interest itself~~, causing serious  
problems in terms of yield, process control, and even  
production cost.

#### BRIEF SUMMARY OF THE INVENTION

10        The present invention relates to a process for  
producing peptides using genetic engineering  
technology. Peptides of interest are expressed with  
the assistance of helper peptides. The addition of  
helper peptides transiently changes the  
15 physicochemical properties of the peptide of interest,  
which enhances yield, promotes efficiency of recovery,  
and purification of the peptide of interest.

#### BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

20        Figure 1 outlines a process for producing the  
peptide of interest using a helper peptide.

Figure 2 is a drawing that shows a process for  
constructing plasmid pG117S4HR6GLP-1.

25        Figure 3 is a drawing that shows a process for  
constructing plasmid pGP117S4HompRHKR.

Figure 4 is a drawing that shows a process for  
constructing plasmid pGP117S4HompRHPR.

Figure 5 is a drawing that shows a process for  
constructing plasmid pGP97ompPR.

30        Figure 6 is a drawing that shows the  
oligonucleotides and the primers used for constructing  
plasmid pGP97ompPR.

Figure 7 is a drawing that shows an amino acid  
sequence of a fusion protein ("GP97ompPR") encoded by  
35 plasmid pGP97ompPR. The underlined part indicates the

amino acid sequence derived from GLP-1(7-37), and the double-underlined part indicates the sequence of a helper peptide. ↓ indicates the cleavage site by the *E. coli* OmpT protease, and the arrow after the double-underline indicates the cleavage site by Kex2 protease.

Figure 8 is a drawing that shows the nucleotide sequence of DNA of a fusion protein ("GP97ompPR") encoded by plasmid pG97ompPR, wherein the region from A of the nucleotide number 1 to T of the nucleotide number 462 encodes the fusion protein of GLP-1(7-37). lac PO represents the promoter/operator region of the *E. coli* lactose operon.

Figure 9 is a photograph of an electrophoresis that shows the culturing of a recombinant *E. coli* cells and an expression of a fusion protein ("GP97ompPR"). In the figure are shown the results of the SDS-polyacrylamide gel electrophoresis of the samples at sampling times "a" through "e." The arrow in the figure indicates the band of the fusion protein.

Figure 10 is a drawing that shows an analysis of the cleavage of a fusion protein ("GP97ompPR") with the *E. coli* OmpT protease present in the inclusion bodies.

Figure 11 is a drawing that shows an amino acid sequence of a fusion protein encoded by plasmid pG117S4HR6GLP-1. In the figure, the underlined part indicates an amino acid sequence derived from GLP-1(7-37), and the double-underlined part indicates an amino acid sequence derived from a helper peptide.

Figure 12 is a drawing that shows an amino acid sequence of a fusion protein encoded by plasmid pGP117S4HompRHKR. In the figure, the underlined part indicates an amino acid sequence derived from GLP-1(7-

37), and the double-underlined part indicates an amino acid sequence derived from a helper peptide.

Figure 13 is a drawing that shows an amino acid sequence of a fusion protein encoded by plasmid pGP117S4HomprHPR. In the figure, the underlined part indicates an amino acid sequence derived from GLP-1(7-37), and the double-underlined part indicates an amino acid sequence derived from a helper peptide.

Figure 14 is a drawing that shows an elution pattern of "RHHGP(G)" from the SP Sepharose Big beads, wherein the starting position of elution is shown by ↓ and the pooled fractions are shown in the figure. Absorbance was measured at 280 nm.

Figure 15 is a drawing that shows an analytical pattern at each purification step in the cleavage process of GLP-1(7-37) from "RHHGP(G)" by Kex2 protease. In the figure, A indicates a reverse-phase pool before cleavage with Kex2 protease, B after cleavage with the Kex2 protease, and C after PorosR2, and 1 indicates "RHHGP(G)" and 2 indicates GLP-1(7-37).

Figure 16 is a drawing that shows the pH dependency of the amidation reaction of "RHHGP(G)."

Figure 17 is a drawing that shows a time course of the conversion of "RHHGP(G)" to "RHHGP-1" in an amidation reaction as measured by an ion exchange HPLC, wherein 1 indicates "RHHGP(G)" and 2 indicates "RHHGP-1." Absorbance was measured at 280 nm.

The analytical condition was as follows:

Column: Poros S/H, 4.6 mm I.D. × 50 mm

Flow rate: 1.6 ml/min

Solution A: 30 mM BR buffer, pH 6.0

Solution B: 30 mM BR buffer, pH 9.0

Equilibration: solution A

Elution: solution B 0% → 100%, linear pH



gradient.

Figure 18 is a drawing that shows the pH dependency of a Kex2 protease processing reaction with "RHHGP-1" as a substrate.

5        Figure 19 is a drawing that shows an elution pattern and a pH gradient created in the purification of GLP-1(7-36)NH<sub>2</sub> by Macrorep High-S. Absorbance was measured at 280 nm.

10       Figure 20 is a drawing that shows the removal of impurities with Macrorep High-S, wherein A indicates the sample loaded to the column, B indicates an analytical HPLC pattern. 1 indicates GLP-1(7-37) and 2 indicates GLP-1(7-36) NH<sub>2</sub>. Absorbance was measured at 280 nm.

15       Figure 21 is a drawing that collectively shows the analytical HPLC patterns of samples in each purification process in the production of GLP-1(7-36)NH<sub>2</sub> through the first cleavage with OmpT and the second cleavage with Kex2 protease. A indicates a reverse-phase HPLC pattern after OmpT, B after SP  
20       Sepharose, C after Kex2 reaction, D after Macrorep High-S, and E after Poros R2. 1 indicates GP97ompPR, 2 indicates the protective peptide, 3 indicates "RHHGP(G)," 4 indicates "RHHGP-1," and 5 indicates  
25       GLP-1(7-36) NH<sub>2</sub>. Absorbance was measured at 280 nm.

The analytical condition was as follows:

Column: YMC Protein RP, 4.6 mm I.D. × 50 mm

Flow rate: 1.0 ml/min

Solution A: 0.1% TFA / 10% acetonitrile

30       Solution B: 0.1% TFA / 60% acetonitrile

Equilibration: solution A

Elution: solution B 44% → 74% /12 min.

Figure 22 is a drawing that shows the pH dependency of the solubility of "RHHGP(G)," "RHHGP-1,"  
35       GLP-1(7-37), and GLP-1(7-36)NH<sub>2</sub>.

Figure 23 is a drawing that shows a suppressing effect on the aggregation of "RHHGP(G)," "RHHGP-1," and GLP-1(7-36)NH<sub>2</sub> by Tween 80, wherein A indicates the suppression of aggregation of "RHHGP(G)" and "RHHGP-1" by Tween 80, and B indicates the suppression of aggregation of GLP-1(7-36)NH<sub>2</sub> by Tween 80.

Figure 24 is a drawing that shows a suppressing effect on the aggregation of GLP-1(7-36)NH<sub>2</sub> by NaCl and temperature, wherein C indicates the suppression of aggregation of "GLP-1(G)" by NaCl and D indicates the suppression of aggregation of GLP-1(7-36)NH<sub>2</sub> by temperature.

[Disclosure of the Invention]

#### DETAILED DESCRIPTION OF THE INVENTION

It is an object of the present invention to provide a process for producing a peptide of interest in an efficient manner by relieving the problems derived from the physicochemical properties of the peptide of interest itself (for example, the problem of low solubility and gelling in the chemical and enzymatic reaction or the purification step in the production processes of the peptide of interest).[, when an efficient] Efficient production of said peptide of interest on an industrial scale using genetic engineering technology is contemplated.

The term "the peptide of interest of the present invention" as used herein means not only the peptide finally obtained but also the production intermediate peptides that are required in the production process thereof.

~~In order to avoid the above mentioned problems, the inventors of the present invention have found a process for producing a peptide of interest in an efficient manner by relieving the problems of the~~

~~peptide of interest~~ The instant inventors alleviate  
prior difficulties in the art experienced when  
producing peptides of interest by adding a helper  
peptide to the peptide of interest. Thus, the process  
5 for producing a peptide claimed in the present  
invention is a method for producing a peptide having  
the desired biological activity, said method  
comprising the steps of:

(1) culturing cells transformed with an  
10 expression vector having the nucleotide sequence  
encoding the peptide of interest that has a helper  
peptide added thereto, or a fusion protein that has a  
protective peptide further added to the peptide of  
interest that has a helper peptide added thereto, and  
15 then harvesting said peptide of interest that has a  
helper peptide added thereto, or said fusion protein,  
from said culture;

(2) in the case wherein a fusion protein is  
obtained in step (1), cleaving off from said fusion  
20 protein the peptide of interest that has a helper  
peptide added thereto and the protective peptide, and  
purifying the peptide of interest that has a helper  
peptide added thereto as desired;

(3) in the case wherein modification is required  
25 for the peptide of interest, subjecting the peptide of  
interest that has a helper peptide added thereto  
obtained in step (1) or step (2) to a modification  
reaction;

(4) cleaving off from the peptide of interest  
30 that has a helper peptide added thereto obtained in  
step (1), step (2) or step (3), the helper peptide and  
the peptide of interest, and purifying the peptide of  
interest as desired; and

(5) purifying the peptide of interest obtained in  
35 step (4) (Figure 1).

### ~~Brief Description of Drawings~~

~~Figure 1 outlines a process for producing the peptide of interest using a helper peptide.~~

5 ~~Figure 2 is a drawing that shows a process for constructing pC117S4HR6GLP 1.~~

~~Figure 3 is a drawing that shows a process for constructing pGP117S4HompRHKR.~~

10 ~~Figure 4 is a drawing that shows a process for constructing pGP117S4HompRHPR.~~

~~Figure 5 is a drawing that shows a process for constructing pGP97ompPR.~~

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~~Figure 7 is a drawing that shows an amino acid sequence of a fusion protein (GP97ompPR) encoded by pGP97ompPR. The underlined part indicates the amino acid sequence derived from GLP 1(7-37), and the double underlined part indicates the sequence of a helper peptide. ↓ indicates the cleavage site by the E. coli OmpT protease, and the arrow after the double underline indicates the cleavage site by Kex2 protease.~~

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30

~~Figure 9 is a photograph of an electrophoresis that shows the culturing of a recombinant E. coli cells and an expression of a fusion protein (GP97ompPR). In the figure are shown the results of~~

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the SDS polyacrylamide gel electrophoresis of the samples at sampling times a through e. The arrow in the figure indicates the band of the fusion protein.

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25 — Figure 14 is a drawing that shows an elution pattern of RHHGP[G] from the SP Sepharose Big beads, wherein the starting position of elution is shown by ↓ and the pooled fractions are shown in the figure. Absorbance was measured at 280 nm.

30 — Figure 15 is a drawing that shows an analytical pattern at each purification step in the cleavage process of GLP-1(7-37) from RHHGP[G] by Kex2 protease. In the figure, A indicates a reverse phase pool before cleavage with Kex2 protease, B after cleavage with the Kex2 protease, and C after PorosR2, and 1 indicates

35

~~RHHGP[G] and 2 indicates CLP 1(7-37).~~

~~—— Figure 16 is a drawing that shows the pH dependency of the amidation reaction of RHHGP[G].~~

5 ~~—— Figure 17 is a drawing that shows a time course of the conversion of RHHGP[G] to RHHGP-1 in an amidation reaction as measured by an ion exchange HPLC, wherein 1 indicates RHHGP[G] and 2 indicates RHHGP-1. Absorbance was measured at 280 nm.~~

~~—— The analytical condition was as follows:~~

10 ~~—— Column: Poros S/H, 4.6 mm I.D. × 50 mm~~

~~—— Flow rate: 1.6 ml/min~~

~~—— Solution A: 30 mM BR buffer, pH 6.0~~

~~—— Solution B: 30 mM BR buffer, pH 9.0~~

~~—— Equilibration: solution A~~

15 ~~—— Elution: solution B 0% → 100%, linear pH gradient.~~

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25 ~~—— Figure 20 is a drawing that shows the removal of impurities with Macrorep High S, wherein A indicates the sample loaded to the column, B indicates an analytical HPLC pattern. 1 indicates CLP 1(7-37) and 2 indicates CLP 1(7-36) NH<sub>2</sub>. Absorbance was measured at 280 nm.~~

30 ~~—— Figure 21 is a drawing that collectively shows the analytical HPLC patterns of samples in each purification process in the production of CLP 1(7-36)NH<sub>2</sub> through the first cleavage with OmpT and the second cleavage with Kex2 protease. A indicates a reverse phase HPLC pattern after OmpT, B after SP~~

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~~Sepharese, C after Kex2 reaction, D after Macrorep  
High S, and E after Poros R2. 1 indicates GP97ompPR,  
2 indicates the protective peptide, 3 indicates  
RHHGP[G], 4 indicates RHHGP 1, and 5 indicates GLP-  
1(7-36) NH<sub>2</sub>. Absorbance was measured at 280 nm.~~

~~— The analytical condition was as follows:~~

~~— Column: YMC Protein RP, 4.6 mm I.D. × 50 mm~~

~~— Flow rate: 1.0 ml/min~~

~~— Solution A: 0.1% TFA / 10% acetonitrile~~

~~— Solution B: 0.1% TFA / 60% acetonitrile~~

~~— Equilibration: solution A~~

~~— Elution: solution B 44% → 74% /12 min.~~

~~— Figure 22 is a drawing that shows the pH  
dependency of the solubility of RHHGP[G], RHHGP 1,  
GLP 1(7-37), and GLP 1(7-36)NH<sub>2</sub>.~~

~~— Figure 23 is a drawing that shows a suppressing  
effect on the aggregation of RHHGP[G], RHHGP 1, and  
GLP 1(7-36)NH<sub>2</sub> by Tween 80, wherein A indicates the  
suppression of aggregation of RHHGP[G] and RHHGP 1 by  
Tween 80, and B indicates the suppression of  
aggregation of GLP 1(7-36)NH<sub>2</sub> by Tween 80.~~

~~— Figure 24 is a drawing that shows a suppressing  
effect on the aggregation of GLP 1(7-36)NH<sub>2</sub> by NaCl and  
temperature, wherein C indicates the suppression of  
aggregation of GLP 1[G] by NaCl and D indicates the  
suppression of aggregation of GLP 1(7-36)NH<sub>2</sub> by  
temperature.~~

#### ~~Embodiments for Carrying Out the Invention~~

The helper peptide according to the present  
invention is a peptide that is used to circumvent the  
problems that occur during [on] industrial production  
[derived from] due to the physicochemical properties  
of the peptide of interest itself. ~~Of the problems on  
industrial production derived from physicochemical  
properties of the peptide of interest itself,~~

Regarding such problems, special attention is paid to the problem in chemical or enzymatic reactions and purification during the production process. ~~of said peptide, such as problems of~~ These problems include:

5 solubility and gelling of the peptide of interest under various conditions [of] undergoing cleavage and modification reactions, [the problem of] sample concentration to be loaded on the column during the column chromatographic process, [the problem of the] 10 elution conditions from the column, [and the] stability after elution, and the like.

The helper peptide of the present invention may be prepared as appropriate depending on the physicochemical properties of the peptide of interest. 15 For example, when the isoelectric point (pI) of the peptide of interest is neutral to weak acid and the optimum pH during the production process is also neutral to weak acid (and thereby the solubility of the peptide of interest under such pH is too low), 20 then the helper peptide is preferably designed so that the isoelectric point [(pI)] of the peptide of interest that has a helper peptide added thereto is 8 to 12, and more preferably 9 to 11. The helper peptide of the present invention may be added to 25 either the [N-terminal or C-terminal] N-terminus or C-terminus of the peptide of interest. The dimension (length) of a helper peptide is preferably 5 to 50 amino acid residues, and more preferably from 5 to not greater than 30 amino acid residues, but it contains 30 at least one basic amino acid or acidic amino acid.

In addition to the above-mentioned GLP-1 derivatives, the peptides of interest that can be produced according to the present invention include, but are not limited to, [the] peptides comprising not 35 greater than 200 amino acid residues. Examples of



such peptides include: adrenocorticotrophic hormone,  
adrenomedullin, amylin, angiotensin I, angiotensin II,  
angiotensin III, A-type natriuretic peptide, B-type  
natriuretic peptide, bradykinin, big gastrin,  
5 calcitonin, calcitonin gene related peptide,  
cholecystokinin, corticotropin releasing factor,  
cortistatin, C-type natriuretic peptide, defesin 1,  
delta sleep-inducing peptide, dynorphin, elafin,  
 $\alpha$ -endorphin,  $\beta$ -endorphin,  $\gamma$ -endorphin, endothelin-1,  
10 endothelin-2, endothelin-3, big endothelin-1, big  
endothelin-2, big endothelin-3, enkephalin, galanin,  
big gastrin, gastrin, gastric inhibitory polypeptide  
(GIP), gastrin releasing peptide, glucagon, glucagon-  
like peptide-2, growth hormone releasing factor,  
15 growth hormone, guanylin, uroguanylin, histatin 5,  
insulin, joining peptide, luteinizing hormone  
releasing hormone, melanocyte stimulating hormone,  
midkine, motilin, neurokinin A, neurokinin B,  
neuromedin B, neuromedin C, neuropeptide Y,  
20 neurotensin, oxytocin, proadrenomedullin N-terminal 20  
peptide, cromogranin A, parathyroid hormone, PTH  
related peptide, peptide histidine-methionine-27,  
pituitary adenylate cyclase activating polypeptide 38,  
platelet factor-4, peptide T, secretin, serum thymic  
25 factor, somatostatin, substance P, thyrotropin  
releasing hormone, urocortin, vasoactive intestinal  
peptide, vasopressin, and the derivatives thereof,  
etc.

As the GLP-1 derivatives, in addition to the  
30 above-mentioned GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub>, there  
can be mentioned peptides having an insulinotropic  
activity in which amino acid residues have been  
substituted for, added to, and/or deleted from the  
peptide comprising 37 amino acid residues of GLP-1,  
35 peptides having an insulinotropic activity in which

amino acids of said peptide have been further modified (for example, an amidated form), and peptides having an insulinotropic activity that are obtained from the combinations thereof.

5           Furthermore, as the GLP-1 derivatives that are suitably produced according to the method of the present invention, those GLP-1 derivatives that have isoelectric points from 4.5 to 9.0 are preferred. More preferably they are GLP-1 derivatives having  
10           isoelectric points from 5.5 to 7.5.

          As the GLP-1 derivatives, in addition to those mentioned above, the following can be mentioned:

          GLP-1(7-34), GLP-1(7-35), GLP-1(7-36), GLP-1(7-34)NH<sub>2</sub>, GLP-1(7-35)NH<sub>2</sub>, and GLP-1(7-37)NH<sub>2</sub>;

15           GLP-1(7-37)-Arg, GLP-1(7-37)-Arg-Arg, GLP-1(7-37)-Lys, GLP-1(7-37)-Lys-Lys, GLP-1(7-37)-Lys-Arg, and GLP-1(7-37)-Arg-Lys, and the C-[terminal]terminus-amidated forms thereof;

          GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino  
20           acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser;

          GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg;

25           GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 34, Lys, of GLP-1 has been substituted with Arg;

          GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 36, Arg, of GLP-1 has been  
30           substituted with Lys;

          GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, and the amino acid at position 26, Lys, has been substituted with Arg;

35           GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino

acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, and the amino acid at position 34, Lys, has been substituted with Arg;

5 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, and the amino acid at position 36, Arg, has been substituted with Lys;

10 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 34, Lys, has been substituted with Arg;

15 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 36, Arg, has been substituted with Lys;

GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 34, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 36, Arg, has been substituted with Lys;

20 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 34, Lys, has been substituted with Arg;

25 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 36, Arg, has been substituted with Lys;

30 GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, the amino acid at position 34, Lys, of GLP-1 has been substituted with Arg, and the

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amino acid at position 36, Arg, has been substituted with Lys;

GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, the amino acid at position 34, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 36, Arg, has been substituted with Lys; and

GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> in which the amino acid at position 8, Ala, of GLP-1 has been substituted with Thr, Gly or Ser, the amino acid at position 26, Lys, of GLP-1 has been substituted with Arg, the amino acid at position 34, Lys, of GLP-1 has been substituted with Arg, and the amino acid at position 36, Arg, has been substituted with Lys.

In the case of GLP-1(7-37) as an example of the peptide of interest, a helper peptide having basic amino acids can be added to GLP-1(7-37) and then can be used for the production of GLP-1(7-37) in order to relieve the problems on purification processes such as the gelling and the solubility of GLP-1(7-37). Thus, by adding the helper peptide to GLP-1(7-37), the isoelectric point (pI = 5.5) of GLP-1(7-37) can be shifted to the alkali side and thereby the hydrophilicity can be increased so as to avoid the problems on purification processes such as aggregation (gelling) in the column. Furthermore, by adding said helper peptide to GLP-1(7-37), it has become possible to isolate a peptide comprising the helper peptide and GLP-1(7-37) at very high purity and high yields in the first chromatographic process, which results in enhanced recovery of GLP-1(7-37), indicating that the use of a helper peptide is very useful in this process.

In the case of GLP-1(7-36)NH<sub>2</sub> as an example of the

peptide of interest, it is necessary to first obtain an intermediate product (said peptide is specifically GLP-1(7-37)) as the peptide of interest since GLP-1(7-36)NH<sub>2</sub> is an amidated peptide. Thus, a helper peptide  
5 having a basic amino acid can be added to GLP-1(7-37), which can be used for the production of GLP-1(7-36)NH<sub>2</sub>. By adding a helper peptide having a basic amino acid, the isoelectric point of GLP-1(7-37) can be shifted to the alkali side and thereby precipitate formation can  
10 be suppressed due to increased solubility of the peptide having a helper peptide added to GLP-1(7-37) at the pH of the amidation enzymatic reaction liquid at a subsequent amidation modification reaction and thereby the recovery and the yield can be increased.  
15 By adding a hydrophilic helper peptide containing a basic amino acid, the solubility of the peptide of interest can also be increased, and furthermore the aggregation of GLP-1(7-37) as the substrate in the amidation modification reaction and GLP-1(7-36)NH<sub>2</sub> as a  
20 product can be avoided, thereby indicating that the addition is very useful in the purification process after the amidation enzymatic reaction.

In any of the above cases, the peptide having a helper peptide added to GLP-1(7-37) has preferably an  
25 isoelectric point of 8 to 12. By subjecting said peptide to the action of a cation ion exchange resin, said peptide can be obtained at high yields (98% or greater).

In order to obtain the peptide of interest from  
30 the peptide that has a helper peptide added thereto, a cleavage site region is introduced that permits chemical or enzymatic cleavage in between the helper peptide and the peptide of interest. For the cleavage site region as well, a cleavage site region having a  
35 high cleavage efficiency is designed depending on the

physicochemical properties owned by the peptide of interest. As the enzymatic and chemical cleavage methods, those described in Methods in ENZYMOLOGY, Vol. 185, Gene Expression Technology (David V. Goeddel ed., published by ACADEMIC PRESS, INC.) can also be  
5 used.

Included within such cleavage methods is: ~~As the chemical cleavage method, there can be mentioned a~~ method in which the [C-terminal] C-terminus end of methionine is cleaved with cyanogen bromide (D. V. Goeddel et al., Proc. Natl. Acad. Sci. U.S.A., Vol. 76, pp. 106-110, 1979), a method in which the -Asp-Pro- sequence is cleaved with formic acid (Biochem. Biophys. Res. Commun., Vol. 40, pp. 1173, 1970), a  
10 method in which -Asn-Gly- sequence is cleaved with hydroxylamine, a method in which the [C-terminal] C-terminus end of trypsin is cleaved with BNPS-skatole or N-chlorosuccinimide, and the like. When methionine is not included in such an amino acid sequence of the  
15 peptide of interest, methionine is introduced at the end of the cleavage site region adjacent to the peptide of interest, and chemical cleavage with cyanogen bromide at the cleavage site region can be carried out.

As an enzymatic cleavage method, it is required to design a cleavage site region that can be specifically recognized as a substrate by an enzyme used for the cleavage treatment. Examples of such methods include the cleavage of: the -X-Gly- sequence  
25 of the X-Gly or the Pro-X-Gly-Pro sequence with collagenase (Proc. Natl. Acad. Sci. U.S.A., Vol. 81, pp. 4692-4696, 1984); the [C-terminal] C-terminus end of Lys of the -Asp-Asp-Asp-Lys- sequence (SEQ ID NO: 1) with enterokinase; the [C-terminal] C-terminus end  
30 of Arg of -Ile-Glu-Gly-Arg- sequence (SEQ ID NO: 2)  
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with blood coagulation factor Xa (Japanese Unexamined Patent Publication (Kokai) No. 61(1986)-135591); the [C-terminal] C-terminus end of Arg of the -Gly-Pro-Arg- sequence with thrombin (Japanese Unexamined Patent Publication (Kokai) No. 62(1987)-135500); the [C-terminal] C-terminus end of -Arg- with trypsin or clostripain; the [C-terminal] C-terminus end of Arg or Lys with endoprotease Arg-C (Nature, Vol. 285, pp. 456-461, 1980); the [C-terminal] C-terminus end of the Lys-Arg, Arg-Arg, or Pro-Arg sequence with *Saccharomyces cereviceae* Kex2 protease and derivatives thereof (Biochem. Biophys. Res. Commun., Vol. 144, pp. 807-814, 1987, Japanese Unexamined Patent Publication (Kokai) No. 1(1989)-199578); the [C-terminal] C-terminus end of Lys with lysyl endopeptidase or endopeptidase Lys-C (Japanese Unexamined Patent Publication (Kokai) No. 61(1986)-275222); the [C-terminal] C-terminus end of Asp or Glu with *Staphylococcus aureus* ("*S. aureus*") V8 protease (Proc. Natl. Acad. Sci. U.S.A., Vol. 69, pp. 3506-3509, 1972); the [C-terminal] C-terminus end of the -Phe-Arg- sequence with kallikrein (Japanese Unexamined Patent Publication (Kokai) No. 62(1987)-248489); Leu-Leu of the -Pro-Phe-His-Leu-Leu-Vla-Tyr- sequence (SEQ ID NO: 3) with renin (Japanese Unexamined Patent Publication (Kokai) No. 60(1985)-262595); the [C-terminal] C-terminus end of the -Glu-Gly-Arg- sequence with urokinase (Japanese Unexamined Patent Publication (Kokai) No. 2(1990)-100685); the [C-terminal] C-terminus end of the Val-Asp-Asp-Asp-Lys sequence (SEQ ID NO: 4) with entero-peptidase (Biotechnology, Vol. 6, pp. 1204-1210, 1988); the [C-terminal] C-terminus end of poly-Gly with lysostaphin (Japanese Unexamined Patent Publication (Kokaki) 1(1989)-160496); the [C-terminal] C-terminus end of Lys-Arg,

Arg-Arg, or Pro-Arg, etc. with *Kluverromyces lactis*)  
(Japanese Unexamined Patent Publication (Kokai) No.  
1(1989)-124390), and the like.

5 In the Examples of the present invention, for  
example, an amino acid sequence (the Lys-Arg, Arg-Arg  
or Pro-Arg sequence) that can be recognized by Kex2  
protease was introduced into the cleavage site region  
and then the peptide of interest was cleaved with the  
enzyme.

10 Depending on the substrate specificity of an  
enzyme used in the cleavage treatment and the amino  
acid sequence of the peptide of interest, therefore,  
it is preferred that one or more of methionine,  
tryptophan, proline, glycine, cysteine, arginine,  
15 lysine, aspartic acid, and glutamic acid is present in  
the amino acid sequence of the cleavage site region.

When modification is required to obtain a final  
peptide of interest (for example, an amidated  
peptide), a modification reaction (for example, an  
20 amidating modification reaction with an amidation  
enzyme) is carried out before or after excising said  
peptide of interest from the peptide of interest  
having a helper peptide added thereto (in this case  
the final peptide of interest is a production  
25 intermediate peptide). Furthermore, efficient  
modification reaction is desired, a modification  
reaction (for example, an amidation modification  
reaction) is performed on the peptide of interest  
having a helper peptide added thereto before excising  
30 the peptide of interest to obtain the final peptide to  
which a helper peptide has been added, and then the  
cleavage site region in between the helper peptide and  
the final peptide of interest is cleaved to obtain the  
final peptide of interest.

35 If the peptide of interest having a helper



peptide added thereto is highly expressed, the peptide of interest can be obtained at high purity and high yields, but in order to obtain larger amounts of the peptide of interest it can be produced by further adding a protective peptide and expressing it as has been conducted in the conventional fusion protein method. Thus, it is also possible to produce the peptide of interest at a high expression level within the host cell as a fusion protein further having a protective peptide added to the peptide of interest having a helper peptide added thereto (the protective peptide may be added to any N-terminus or C-terminus [of the N-terminal or C-terminal] of the peptide of interest having a helper peptide added thereto).

The protective peptide for use in the production process of the present invention is not specifically limited and [the] conventionally-used peptides can be used as appropriate with modifications. For example, in Japanese Unexamined Patent Publication (Kokai) No. 54(1979)-145289, fragments of the amino acid sequences from  $\beta$ -galactosidase derived from *E. coli* can be used as a protective peptide. The amino acid sequences of said enzyme are known to a person skilled in the art, and peptide fragments derived from  $\beta$ -galactosidase have been widely used as the protective peptide in the fusion protein method by persons skilled in the art. In the production processes of the present invention, peptide fragments that were obtained by appropriate modification of the amino acid sequences of  $\beta$ -galactosidase can be used as the protective peptide by taking into account the characteristics of the peptide of interest having a helper peptide added thereto. It is also possible to chemically synthesize a DNA base sequence encoding the amino acid sequence

of the protective peptide.

For fusion proteins having a protective peptide, it is preferred to devise and obtain different isoelectric points for each of the protective peptide, the peptide of interest having a helper peptide added thereto constituting said fusion protein, and said fusion protein, in order to enhance the fragment resolving ability during the chromatographic processes after the cleavage treatment reaction. The same is also true for the helper peptide and the peptide of interest.

When a protective peptide is added, as mentioned above, it is also necessary to provide a cleavage site region [in] between the peptide of interest having a helper peptide added thereto and the protective peptide, but it is possible to provide a cleavage site region having a preferable high cleavage efficiency as appropriate according to the above-mentioned strategy. However, when it is intended to obtain the peptide of interest through a fusion protein having the protective peptide to the peptide of interest having a helper peptide added thereto, a multi-step method of cleaving the fusion protein is required at each site since a plurality of cleavage site regions have been provided. In this case, the first cleavage treatment is carried out in the cleavage site region in between the peptide of interest having a helper peptide added thereto and the protective peptide, and then a cleavage treatment is carried out in the cleavage site region in between the helper peptide and the peptide of interest thereby to obtain the peptide of interest.

In order to confirm the wide applicability of the above production process, processes were examined, in the chemical or enzymatic cleavage of each cleavage site region and it was confirmed that any method of

cleavage treatment can be carried out. Representative examples of the site-specific cleavage method of peptide at such cleavage site regions include:

5 (1) a method in which the absence of cysteine residues in the amino acid sequences of the protective peptide and the peptide of interest is utilized, and cysteine is introduced [in] between the peptide of interest having a helper peptide added thereto and the protective peptide, which is then cyanized, subjected  
10 to an alkali treatment, and the fusion protein is specifically cleaved at the site;

(2) a method in which cleavage at each cleavage site region is carried out with the same enzyme, but by using different amino acid sequences at the enzyme  
15 recognition sites, the reaction condition for one cleavage site region is designed so as not to permit cleavage at another cleavage site region; and

(3) a method in which cleavage at each cleavage site region is carried out with the same enzyme, but  
20 by modifying amino acids in the cleavage site region (cleavage site region 2) present in between the helper peptide and the peptide of interest, the reaction condition for cleavage of the other cleavage site region (cleavage site region 1) present in between the  
25 peptide of interest having a helper peptide added thereto and the protective peptide is designed so as not to cause cleavage at cleavage site region 2, and after cleavage at cleavage site region 1, the peptide of interest having a helper peptide added thereto is  
30 purified, and then the modified amino acid is modified again so that cleavage site region 2 can be made possible to be cleaved with the above enzyme.

Host cells that can be used in the present invention are not specifically limited and the  
35 conventionally-used prokaryotic cells or eukaryotic

cells, for example microorganism cells such as *E. coli*, yeasts or animal cells that can suitably express, using an expression vector having a nucleotide sequence encoding, a peptide of interest having a helper peptide added thereto can be chosen as appropriate and used. Furthermore, other elements required for high expression such as a promoter, a terminator, and splice sites are also chosen from the conventionally used ones and used as appropriate.

In the production process of the peptide of interest of the present invention, the following describes a case wherein the peptide of interest is GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub>.

The fusion protein (referred to hereinafter as GP97ompPR) encoded by the GLP-1(7-37) expression vector (referred to hereinafter as pGP97ompPR) is a fusion protein that has a peptide (referred to hereinafter as "RHHGP(G)") having a helper peptide added thereto, said helper peptide containing a basic peptide region in the N-terminal end of GLP-1(7-37), and that has an *E. coli*  $\beta$ -galactosidase derivative ( $\beta$ -gal97S) added to the N-terminus [N-terminal] end of [RHHGP(G)] "RHHGP(G)" as a protective peptide. A cleavage site region has been introduced in between the protective peptide and [RHHGP(G)] "RHHGP(G)" and between the helper peptide of [RHHGP(G)] "RHHGP(G)" and GLP-1(7-37). Each of the regions has amino acid sequences responsible for substrate specificity so that one cleavage site region may be cleaved with an endogenous OmpT protease derived from *E. coli*, and the other cleavage site region may be cleaved with Kex2 protease (Japanese Patent No. 2643968, and Japanese Unexamined Patent Publication (Kokai) 10(1998)-229884, and the like).

For GP97ompPR, the isoelectric point of each of

$\beta$ -gal97S or [RHHGP[G]] "RHHGP(G)" and GP97ompPR has been designed to be different from each other, in order to enhance the fragment resolving ability in the chromatographic process after the cleavage treatment reaction. For example, in Examples described below, the isoelectric point of GP97ompPR has been designed to be 5.95, that of  $\beta$ -gal97S to be 4.60, and that of [RHHGP[G]] "RHHGP(G)" to be 10.09.

Then, *E. coli* (W3110/pG97ompPR) transformed with pGP97ompPR was cultured to express GP97ompPR. GP97ompPR was highly expressed as an insoluble protein in the cells and was accumulated in the inclusion bodies. The final cell density reached to 180 at OD660 nm.

After disrupting the cells, GP97ompPR was solubilized with urea, and the cleavage site region in between the protective peptide  $\beta$ -gal97S and the peptide of interest [RHHGP[G]] "RHHGP(G)" having a helper peptide added thereto in GP97ompPR was cleaved with the endogenous OmpT protease existing in the inclusion body. OmpT protease specifically cleaved said cleavage site region with a cleavage efficiency of 85%.

Next, in order to separate  $\beta$ -gal97S and [RHHGP[G]] "RHHGP(G)" and to remove urea, a cation exchange chromatography was carried out. Since the isoelectric points of non-cleaved GP97ompPR [(pI = 5.95)] having a pI of 5.95 and that of  $\beta$ -gal97S [(pI = 4.60)] having a pI of 4.60 were both in the acid side they do not adsorb to the column, and [RHHGP[G]] "RHHGP(G)" having a pI of 10.09 [(pI = 10.09)] was adsorbed and eluted. Through this one step of column treatment alone, [RHHGP[G]] "RHHGP(G)" at a purity of 99% was obtained. The fact that the first stage of

column procedures during the production process provides [RHHGP[G]] "RHHGP(G)" at such a high purity and high yield is very useful from the viewpoint of industrial large-scale production.

5           The cleavage site region in between the helper peptide and the peptide of interest present in [RHHGP[G]] "RHHGP(G)" (1.0 g) was cleaved using Kex2 protease. In the reaction condition described in Examples below, the reaction proceeded at a cleavage  
10 efficiency of 95%. The recovery was 90%.

Subsequently, a reverse-phase chromatography was carried out to further purify GLP-1(7-37). The recovery of this process was 80%, the final yield of the overall processes was about 64% and 0.72 g of GLP-  
15 1(7-37) having a purity of 98% was obtained. Since the amount of the culture broth used for purification was about 0.36 liter, this means that about 2.0 g was obtained per liter of the culture broth. The recovery and the yield are both very high and thereby have  
20 proved the usefulness of the production method of the present invention that employs a helper peptide. Thus, the production process of the present invention enables full realization of the production of the peptide of interest on an industrial scale.

25           Concerning the production of GLP-1(7-37) using the production process of the present invention, the yield at each step is shown in Table 2-B below.

As can be seen from Table 2-B, it was demonstrated that the yield at each step is very high  
30 and the finally recovery of 64% is very high.

Furthermore, when the peptide of interest is GLP-1(7-36)NH<sub>2</sub>, an amidation modification reaction is required since the peptide is an amidated peptide. The peptide can be obtained as follows:

35           [RHHGP[G]] "RHHGP(G)" obtained as mentioned above

was subjected to an amidation modification reaction using an amidation enzyme (B. B. R. C., Vol. 150, pp. 1275-1281, 1988, EP299790A, and the like). Under the reaction condition in the Examples described below, no aggregation or gelling of [RHHGP[G]] "RHHGP(G)" as the enzyme substrate and the reaction product, amidated GLP-1(7-36)NH<sub>2</sub> having a helper peptide added thereto (referred to hereinafter as "RHHGP-1") occurs, and "RHHGP-1" could be produced at a high reaction rate of 98% (a recovery of 95%). These results demonstrated the usefulness of the helper peptide in the amidation enzymatic reaction conducted with a substrate of [RHHGP[G]] "RHHGP(G)." [.]

After the amidation modification reaction, the cleavage site region in between the helper peptide and the peptide of interest present in "RHHGP-1" was cleaved using Kex2 protease. Under the reaction condition in Examples described below, the reaction proceeded at a cleavage efficiency of 95% (a recovery of 90%) or greater.

In order to further purify GLP-1(7-36)NH<sub>2</sub>, a cation exchange chromatography and then a hydrophobic chromatography were carried out. The final yield of the overall process was about 50% and 13.5 g of GLP-1(7-36)NH<sub>2</sub> with a purity of 98% was obtained. Since the amount of the culture broth used for purification was about 8 liters, this means that about 1.68 g was obtained per liter of the culture broth. The recovery and the yield are both very high and thereby it was demonstrated that the production method of the present invention that employs a helper peptide is very useful. Thus, the production process of the present invention enables full realization of the production of the peptide of interest on an industrial scale.

One object of the present invention on the

production of GLP-1(7-36)NH<sub>2</sub> is to relieve aggregation and increase solubility at the time of reaction of modification enzymes such as an amidation enzyme described above through a peptide comprising GLP-1(7-37) having a helper peptide added thereto. In order to further investigate the usefulness of the present invention, [RHHGP[G]] "RHHGP(G)", "RHHGP-1", GLP-1(7-37), and GLP-1(7-36)NH<sub>2</sub> were purified and the pH dependency of the solubility of each peptide was examined. As a result, GLP-1(7-37) was shown to have a low solubility in the range of pH 5.0 to pH 7.0 as expected. On the other hand, [RHHGP[G]] "RHHGP(G)" had a high solubility in the range of from pH 4.0 to about pH 6.0. The result confirmed that the substitution of [RHHGP[G]] "RHHGP(G)" having a helper peptide for GLP-1(7-37) as an enzyme substrate is useful since the amidation enzyme reaction is carried out in the weak acid region.

In the experiment that investigated the pH dependency of solubility of each peptide, [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" have shown a sudden reduction in solubility at about pH 6.0 and about pH 6.4, respectively. GLP-1(7-36)NH<sub>2</sub> formed precipitates or microcrystals with time. Thus, it is estimated that substances capable of increasing the solubility of [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" in the neutral to weak alkali region and substances capable of increasing the solubility of the peptide of interest in the weak acid to weak alkali region would be very useful in the production process.

Thus, after an intensive study to search such substances, the inventors of the present invention have found that aggregation can be effectively prevented by adding to the reaction mixture a surfactant (for example, the addition of 0.1% Tween



80) in the case of [RHHGP[G]] "RHHGP(G)" and "RHHGP-1," a surfactant (for example, the addition of 0.3% or greater of Tween 80) and/or a salt (for example, 100 mM or greater of NaCl) in the case of GLP-1(7-36)NH<sub>2</sub>, and could prove the usefulness thereof by introducing it to the production method of the present invention.

For the production process of GLP-1(7-36)NH<sub>2</sub> using the production method of the present invention, the yield of each step obtained in the purification of GLP-1(7-36)NH<sub>2</sub> on a 10 g scale is collectively shown in Table 1 (20 liters of the producing organism W3110/pGP97ompPR was cultured and an 8 liter portion of the culture broth was used for the purification).

Table 1  
Summary of production steps of GLP-1(7-36)NH<sub>2</sub>

Steps	Amount of GLP-1(7-37) / GLP-1(7-36)NH <sub>2</sub> (g)	Yield of Unit process (%)	Overall recovery (%)
Culture (corresponding to 8 L culture broth)	26.87	100	100
OmpT reaction	22.95	85.4	85.4
SP sepharose chromato.	20.22	98.8	76.2
Amidation enzyme reaction	20.70	100	77.0
Kex2 enzyme reaction	18.70	90.4	69.6
MacroPrep HS chromato.	16.78	93.7	62.4
Poros R2 chromato.	13.48	80.4	50.2

The amount of GLP-1(7-37) is shown for the process from the culturing to the amidation enzyme reaction, and the amount of GLP-1(7-36)NH<sub>2</sub> is shown for the process from the Kex2 step to the Poros R2 chromatography step. The amount of GLP-1(7-37) / GLP-1(7-36)NH<sub>2</sub> was calculated from the ratio of the peak area of HPLC to the number of amino acids.

As is evident from Table 1, the yield of a unit process in each step is very high, and the overall yield was shown to be as very high as about 50%. Thus, it is clear that the process for producing peptides of the present invention can be applied to

the production of GLP-1(7-36)NH<sub>2</sub> and that it can be scaled up to an industrial production level.

Furthermore, since the yield of the unit process in the process of cleavage treatment reaction with OmpT protease and Kex2 protease is 85% and 90.4%, respectively, it was confirmed that each cleavage site region designed had amino acid sequences very suitable for the cleavage treatment reaction with the enzyme.

As hereinabove described, it was shown, as an example of the process for producing a GLP-1 derivative having an insulintropic activity, that the present invention can relieve the problems in production processes due to the intrinsic physicochemical properties of the peptide of interest. The problems on production due to the physicochemical properties of the GLP-1 derivatives represented by GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> that were specifically described can be overcome using the production method according to the present invention, and, needless to say, the present invention is also useful for the production of GLP-1 derivatives.

The above-mentioned production of GLP-1 derivatives can be effected by a method using a fusion protein having a protective peptide added thereto. However, when the peptide of interest is obtained from said fusion protein through the peptide of interest having a helper peptide added thereto, multiple cleavage site regions are provided, and therefore a multi-step method of cleaving the fusion protein will be required.

Thus, the inventors have investigated on methods for chemically or enzymatically cleaving each cleavage site region, and have confirmed that it is possible by a method other than the one that employs the cleavage with *E. coli* OmpT protease confirmed in particular in

an Example described below. Representative examples of the site-specific cleavage method of peptide at such cleavage site regions include:

5 (1) a method in which the absence of cysteine residues in the amino acid sequences of the protective peptide and the peptide of interest is utilized, and cysteine is introduced at the [C-terminal] C-terminus of the protective peptide, which is then cyanized, subjected to an alkali treatment to specifically  
10 cleave the fusion protein at said [N-terminal] N-terminus of the cysteine;

(2) a method in which cleavage at each cleavage site region is carried out with Kex2 protease, but by using different amino acid sequences at the enzyme  
15 recognition sites, the reaction condition for one cleavage site region is designed so as not to permit cleavage at another cleavage site region; and

(3) a method in which cleavage at each cleavage site region is carried out with Kex2 protease, but by  
20 modifying amino acids in one cleavage site region (cleavage site region 2) , the reaction condition for cleavage of the other cleavage site region (cleavage site region 1) is designed so as not to cause cleavage at the former cleavage site (cleavage site region 2),  
25 and after cleavage at the latter cleavage site region (cleavage site region 1), the peptide comprising a helper peptide and the peptide of interest is purified, and then the modified amino acid is modified again so that the former cleavage site region  
30 (cleavage site region 2) can be made possible to be cleaved with Kex2 protease.

#### EXAMPLES

35 With a GLP-1 derivative as the peptide of interest, the present invention will now be explained

in further detail with reference to the following Examples.

As a production process when the peptide of interest is GLP-1(7-37), as described above, the peptide can be produced by expressing a fusion protein comprising the protective peptide and GLP-1(7-37) in a conventional method, and then by excising GLP-1(7-37) directly from said protein. However, this method had problems on the purification process in which the gelling or aggregation of GLP-1(7-37) occurred during purification due to its physicochemical properties, which resulted in such problems as extreme reduction in recovery and incapacitation of regeneration of the resin. This drawback could be circumvented by changing the physicochemical properties of GLP-1(7-37) by adding a helper peptide thereto. The method for producing said peptide is now explained more specifically below.

#### Example 1. Construction of plasmid

An expression plasmid pGP97ompPR that encodes a fusion protein (GP97ompPR) designed for the production of GLP-1(7-37) was prepared through the 4 steps shown below. The restriction enzyme treatment, the ligation reaction, the phosphorylation of the 5'-end, and PCR conditions were carried out according to conventional methods.

##### (1) Step 1 Construction of pG117S4HR6GLP-1

In order to design GP97ompPR that can be cleaved with *E. coli* OmpT protease, R6 synthetic DNA (see Figure 6) encoding the amino acid sequence R6 (see Figure 2) having the Arg-Arg sequence, a recognition sequence for OmpT protease, was inserted into a StuI site of pG117S4HGP (see Japanese Unexamined Patent Publication (Kokai) No. 9(1997)-296000) to construct pG117S4HR6GLP-1 (Figure 2).

(2) Step 2 Construction of pGP117S4HompRHKR

In order to further enhance the efficiency of cleavage by *E. coli* OmpT protease, the sequence of the R6 portion was changed. A 3.2 kb fragment (fragment A) obtained by cleaving pG117S4HR6GLP-1 with Nsi I and Hind III was ligated to a 0.2 kb fragment (fragment B) obtained by cleaving PG117S4HR6GLP-1 with BamH I and Hind III and L1 synthetic DNA (see Figure 6) encoding a portion of the amino acid sequence L1 (see Figure 3) having the Arg-Arg sequence, a recognition sequence for OmpT protease, to construct pGP117S4HompRHKR (Figure 3).

(3) Step 3 Construction of pGP117S4HompRHPR

In order to make the *E. coli* OmpT protease recognition sequence and the Kex2 protease recognition sequence different from each other in the cleavage site region, Lys-Arg in the Kex2 protease recognition sequence was replaced with Pro-Arg. P1 primer and P2 primer (see Figure 6) were synthesized, which were subjected to PCR using pGP117S4HompRHKR as the template to construct a 0.1 kb DNA fragment. After treating the thus obtained DNA fragment with Bgl II and Sph I, it was ligated to a 3.2 kb fragment (fragment C) obtained by cleaving pGP117S4HompRHKR with Bgl II and Hind III and a 0.2 kb fragment (fragment D) obtained by cleaving pGP117S4HompRHKR with Sph I and Hind III to construct pGP117S4HompRHPR (Figure 4).

(4) Step 4 Construction of pGP97ompPR (Figure 5)

In order to further reduce the size of the protective peptide portion, pGP97ompPR was constructed. P3 and P4 primers (see Figure 6) were synthesized, which were subjected to PCR using pGP117S4HompRHPR as the template to construct a DNA fragment. After treating the thus obtained DNA

fragment with Pvu II and Nsi I, it was ligated to a 3.2 kb fragment obtained by cleaving pGP117S4HompRHPR with Pvu II and Nsi I to construct pGP97ompPR.

The amino acid sequence of the fusion protein (GP97ompPR) encoded by the thus constructed plasmid pGP97ompPR is shown in Figure 7, and the DNA nucleotide sequence encoding said amino acid sequence is shown in Figure 8.

Example 2. Expression of fusion protein  
(GP97ompPR)

E. coli strain W3110 having pGP97ompPR was cultured in a culture medium (20 L, pH 7.0) containing 4 g/l  $K_2HPO_4$ , 4 g/l  $KH_2PO_4$ , 2.7 g/l  $Na_2HPO_4$ , 0.2 g/l  $NH_4Cl$ , 1.2 g/l  $(NH_4)_2SO_4$ , 4 g/l yeast extract, 2 g/l  $MgSO_4 \cdot 7H_2O$ , 40 mg/l  $CaCl_2 \cdot 2H_2O$ , 40 mg/l  $FeSO_4 \cdot 7H_2O$ , 10 mg/l  $MnSO_4 \cdot nH_2O$ , 10 mg/l  $AlCl_3 \cdot 6H_2O$ , 4 mg/l  $CoCl_2 \cdot 6H_2O$ , 2 mg/l  $ZnSO_4 \cdot 7H_2O$ , 2 mg/l  $Na_2MoO_4 \cdot 2H_2O$ , 1 mg/l  $CuCl_2 \cdot 2H_2O$ , 0.5 mg/l  $H_3BO_4$ , and 10 mg/l tetracycline in a 30-liter jar fermenter at a temperature of 32°C for 12 hours and thereafter at 37°C for 24 hours while adding glucose. The pH of the medium was controlled at pH 7.0 by adding a 28% ammonium solution. Figure 9 is the result of changes in cell density (OD660), and the expression of the fusion protein (GP97ompPR) at the time of sampling that were analyzed with 16% SDS-PAGE. GP97ompPR was expressed as the inclusion body and accounted for 30% or more of the total cellular protein at the end of culturing.

After culturing, the culture broth was homogenized at 500 Kg/cm<sup>2</sup> using a Manton-Gaulin homogenizer (15M-8TA), and then was centrifuged to collect the precipitate fraction (inclusion body). Then, in order to wash the obtained precipitate, an equal amount of deionized water was added to the

culture broth. After suspension, centrifugation was repeated to collect the precipitate. The washing process was repeated again and finally the obtained precipitate was suspended in a suitable amount of deionized water.

Example 3. Processing of GP97ompPR with *E. coli* OmpT protease

The obtained suspension of the inclusion body was diluted to give an OD660 value of 1000. From the liquid a 1000 ml portion was taken, to which 250 ml of 1M Tris-HCl for which pH has not been adjusted, 10 ml of 0.5 M EDTA (pH 8.0), and 1200 g of powdered urea was added, and then deionized water was added thereto to give a final volume of 5000 ml. Then HCl was used to adjust pH at 7.5 and the liquid was heated to 37 °C for 2 hours. This procedure initialed the action of *E. coli* OmpT protease present in the inclusion body, and GP97ompPR was cleaved and [RHHGP[G]] "RHHGP(G)" was released. Figure 10 is the result of the [RHHGP[G]] "RHHGP(G)" that was excised from GP97ompPR and then was analyzed with reverse phase HPLC. The analysis used a YMC PROTEIN-PR column, a 10% acetonitrile solution containing 0.1% trifluoroacetic acid as solution A and a 70% acetonitrile solution containing 0.095% trifluoroacetic acid as solution B, with a flow rate of 1 ml/min and a linear gradient of solution B from 44% to 70% in 13 minutes. This procedure cleaved 85% of GP97ompPR and at the end of the reaction a peak corresponding to [RHHGP[G]] "RHHGP(G)" was obtained (Figure 21 A).

The excision of GLP-1(7-37) having a helper peptide added thereto from the fusion protein by the cleavage treatment with *E. coli* OmpT protease was not limited to the fusion protein derived from pGP97ompPR, and was also possible for fusion proteins derived from

pG117S4HR6GLP-1, pGP117S4HompRHKR and pGP117S4HompRHPR constructed in Example 1. The amino acid sequences of these fusion proteins derived from plasmids are shown in Figures 11, 12, and 13.

5        Example 4. Purification of [RHHGP[G]] "RHHGP(G)"

After the *E. coli* OmpT protease reaction, urea and Tween 80 were added to a concentration of 7M and 0.1%, respectively, and pH was adjusted to 8.0 with NaOH. The mixture was then centrifuged to obtain a  
10        supernatant. A column charged with SP-Sepharose BigBeads (Amersham Pharmacia Biotechnology) was equilibrated with 100 mM Tris HCl, pH 8.0, and then 20 mM Tris HCl, pH 8.0 / 5 M urea / 0.1% Tween 80. After the above supernatant was added to the equilibrated  
15        column, the column was washed in the same equilibration solution, and then was washed with 0.2 M NaCl / 20 mM Tris HCl, pH 8.0 / 0.1% Tween 80 and eluted with 0.5 M NaCl / 20 mM Tris HCl, pH 8.0 / 0.1% Tween 80 (Figure 14).

20        The purity of [RHHGP[G]] "RHHGP(G)" in the eluate was as high as 98%. The reason why such a high purity peptide could be obtained by a simple procedure of the addition of a helper peptide followed by a stepwise elution from a low pressure chromatography column is  
25        that at the time of designing a helper peptide, the introduction of hydrophilic amino acids and isoelectric points were taken into account for purification with an ion exchange column.

Example 5 shows that the above result has greatly  
30        contributed to saving labor during the purification process after the present step.

Example 5. Excision and purification of GLP-1(7-37) from [RHHGP[G]] "RHHGP(G)"

Processing with Kex2 protease was carried out  
35        using [RHHGP[G]] "RHHGP(G)", purified in Example 4, as



the following reaction mixture: 5.0 mg/ml RHHGP[G], 20 mM Tris HCl, 0.1% Tween 80, 0.3 M NaCl, pH 8.0, 2.0 mM CaCl<sub>2</sub>, and 8000 units/ml Kex2 protease (about 1.0 mg/L). A reaction rate of 95% was obtained in one hour (Figure 15-B). During the reaction, no precipitate formation of GLP-1(7-37) was observed.

Immediately after the enzyme reaction, ammonium acetate was added to a final concentration of 10 mM and was adjusted to pH 4.5 with HCl. To a Poros R2 column equilibrated with 10 mM ammonium acetate, pH 4.5, the above solution was loaded to a concentration of 10 mg GLP-1(7-37) equivalents / ml resin. After the column was washed in the same equilibration liquid and then in 0.2% acetic acid / 10% acetonitrile, and was eluted with 0.2% acetic acid / 40% acetonitrile. After removing acetonitrile, a lyophilized product was obtained. The recovery of GLP-1(7-37) was 80% with a purity of 99% (Figure 15-C).

Comparison of the method (see, for example, Japanese Unexamined Patent Publication (Kokai) 9(1997)-296000) for excising GLP-1(7-37) directly from a fusion protein comprising the protective peptide and the peptide of interest with the method of the present invention is shown in Table 2.

Table 2  
Comparison of GLP-1(7-37) production process

<u>A</u>			
<u>Process</u>	<u>Purity (%)</u>	<u>Yield of unit process (%)</u>	<u>Overall recovery (%)</u>
Kex2 enzyme reaction	15	-	100
Acid precipitation treatment	-	68	68
Filtration	75	88	60
Cation exchange chromatography	95	95	57
Reverse phase chromatography	99	72	41

B

Process	Purity (%)	Yield of unit process (%)	Overall recovery (%)
OmpT reaction	24	-	100
Filtration	24	90	90
Cation exchange chromatography	99	99	89
Kex2 enzyme reaction	96	90	80
Reverse phase chromatography	98	80	64

A is based on a method of excising GLP-1(7-37) directly from a fusion protein comprising a protective peptide and the peptide of interest. B is based on a method of excising GLP-1(7-37) from a fusion protein having a protective peptide further added to the peptide of interest having a helper peptide added thereto.

By using [RHHGP[G]] "RHHGP(G)" as an intermediate, GLP-1(7-37) having a purity of 99% was obtained at ease and at a high yield. Since HPLC is not used in the purification of the present invention, it is, needless to say, easy to scale up to an industrial scale.

The following is an example wherein a peptide of interest requires modification. Since GLP-1(7-36)NH<sub>2</sub> is an amidated peptide, an amidation modification reaction is required.

Example 6. An amidation modification reaction of GLP-1(7-37) having a helper peptide added thereto

[RHHGP[G]] "RHHGP(G)" obtained in Example 4 was converted to "RHHGP-1" using an amidation enzyme. In order to determine the reaction condition for the case wherein [RHHGP[G]] "RHHGP(G)" was used as a substrate, optimization was carried out for pH, temperature, the concentration of copper sulfate, catalase concentration, substrate concentration, L-ascorbic acid concentration, and the concentration of the amidation enzyme in a reaction volume of 0.5 ml.

Separation of [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" and analysis thereof were carried out using an ion exchange HPLC column (Poros S/H, Perceptive Biosystems) in the presence of 30 mM Britton-Robinson buffer (referred to hereinafter as the BR buffer) excluding barbital at a pH gradient elution (6.0 to 9.0).

The optimum pH of the reaction condition was 5.0 to 5.5 (Figure 16). The optimum reaction condition was 10 mM sodium acetate, pH 5.0, 5.0  $\mu$ M copper sulfate, 0.5 g/l L-ascorbic acid, 1  $\mu$ M/ml catalase, 5.0 mg/ml [RHHGP[G]] "RHHGP(G)", a temperature of 32  $^{\circ}$ C, and 1500 units/ml amidation enzyme. To this condition was added Tween 80 (0.1%) that was found to have an aggregation suppression effect in Example 11 below, 5 liters of the [RHHGP[G]] "RHHGP(G)" solution was reacted in the above condition, and the reaction was stopped by adding EDTA. As a result of the reaction under the present condition, [RHHGP[G]] "RHHGP(G)" was converted with "RHHGP-1" with a reaction rate of 98% or greater in one hour (Figure 17).

Example 7. Excision of GLP-1(7-36)NH<sub>2</sub> from "RHHGP-1" with Kex2 protease

The processing reaction with Kex2 protease shows a pH dependency and activity change depending on the sequence of the portion that serves as the substrate (EP794255A). Accordingly, the optimization of pH, calcium chloride concentration and the amount of the enzyme added was carried out in a reaction volume of 0.5 ml. In the case of "RHHGP-1," the reaction was shown to reach the maximum at pH 8.0 (Figure 18). The optimum reaction condition when "RHHGP-1" was used as the substrate was set at 10 mM Tris-HCl, pH 8.0, 1 mM calcium chloride, 8,000 units/ml Kex2 protease and a

reaction temperature of 30°C to 32°C. From the results in Example 11 below, aggregation could be avoided by setting the NaCl concentration in the reaction mixture at 0.1 M or greater and by further  
5 adding Tween 80 to a concentration of 0.1%.

By reacting the sample solution after the amidation reaction in Example 6 at 30°C under the present condition for 2 hours, a reaction rate of 95% or greater was obtained (Figure 21C). During the  
10 reaction, no precipitate formation of GLP-1(7-36)NH<sub>2</sub> was observed.

Example 8. Purification of GLP-1(7-36)NH<sub>2</sub>

In order to remove impurities at minute amounts, a pH gradient elution using a cation exchange resin  
15 (MacroPrep High-S, BioRad) was used to separate GLP-1(7-36)NH<sub>2</sub> therefrom. The column was equilibrated with 20 mM BR buffer, pH 4.5 / 20 mM NaCl / 0.3% Tween 80. The composition of the sample solution was set at 0.3 M NaCl / 0.3% Tween 80, pH 4.5. The sample solution  
20 was added to the column, which was washed in the equilibration solution. Using the equilibration solution (solution A) and the elution solution (solution B; pH 7.0 with the same composition as the equilibration solution), GLP-1(7-36)NH<sub>2</sub> was eluted in a  
25 linear gradient from 50% solution B to 100% solution B (Figure 19) to make the proportion of the impurities less than 0.5% (Figure 20). In this process, most of the reagents added up to Example 7, unreacted products, and trace amounts of impurities were  
30 removed, and GLP-1(7-36)NH<sub>2</sub> with a purity of 98% or greater was obtained (Figure 21D). The pooled solution had a pH of 4.5 and was stored at 4 °C.

Example 9. Final purification of GLP-1(7-36)NH<sub>2</sub>

In the above Example 8, GLP-1(7-36)NH<sub>2</sub> with a  
35 purity of 98% or greater was obtained. When used as a

pharmaceutical drug, however, not only the purity of the peptide of interest is important, but the contamination of non-peptide endotoxin must be avoided. Accordingly, in attempts to remove endotoxin etc., a preparative reverse phase HPLC column that is often used for the final purification of peptide drugs was used. But there were times when the aggregation and/or gelling of GLP-1(7-36)NH<sub>2</sub> occurred in the column. It was expected that the tendency of GLP-1(7-36)NH<sub>2</sub> to aggregate easily provides a major risk factor in scaling up.

On the other hand, although the hydrophobic chromatographic resin adsorbs substances utilizing the hydrophobicity thereof as with the reverse phase chromatographic resin, it is generally low in the density of functional groups and has an adsorption volume of 5 to 15 mg/ml resin. However, there are many kinds of carriers and many kinds of functional groups, and therefore it is possible to choose peptides that give a high recovery rate even when they have a tendency to easily aggregate like GLP-1(7-36)NH<sub>2</sub>. After investigating various functional hydrophobic chromatographic resins, we have found that resins comprising hydrophilic carriers having butyl groups, isobutyl groups, hexyl groups or phenyl groups resins or polystyrene resins represented by Poros R2 are suitable. One example of such a resin, Poros R2 (Perceptive Systems) is shown below.

A maximum adsorption volume of the resin for GLP-1(7-36)NH<sub>2</sub> is about 12 mg/ml resin, which is higher than the other hydrophobic chromatographic resins, and which results in a higher concentration of GLP-1(7-36)NH<sub>2</sub> during elution, suggesting that the resin is suitable for lyophilization.

A 800 ml column was equilibrated with 10 mM

ammonium acetate, pH 4.5, to which was added the sample solution of Example 8. After washing with the equilibration solution, the column was further washed with 0.2% acetic acid, and was eluted with 0.2% acetic acid / 40% acetonitrile. The recovery of GLP-1(7-36)NH<sub>2</sub> was 80% and the purity was 98% (Figure 21E). The product only contains volatile acids at a low concentration and therefore could be easily lyophilized after removal of acetonitrile. After reconstituting the lyophilized product, endotoxin was measured by the gelling method (Limulus ES-II test, Wako Pure Chemicals Co., Ltd.) and was found to be below the detection limit (0.03 µ/mg or lower). The fact that the column procedure described in the above method permits the purification of high yield and high purity GLP-1(7-36)NH<sub>2</sub> and enables elution by a solvent that can be lyophilized is, needless to say, industrially very useful.

Example 10. Alleviation of aggregation using a helper peptide

One object of the production method of the present invention that employs a helper peptide is to alleviate the aggregation property that is a characteristics derived from the physicochemical properties of the peptide of interest, which has been a problem in the conventional production method, and therefore it is necessary to investigate the presence of the alleviation. In the present Example, therefore, it was investigated whether the aggregation property of a peptide comprising a helper peptide and GLP-1(7-37) and a peptide comprising a helper peptide and GLP-1(7-36)NH<sub>2</sub> has been improved compared to that of GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub>.

First, [RHHGP[G]] "RHHGP(G)" was purified and the improvement of the aggregation property compared to

that of GLP-1(7-37) was investigated and at the same time substances that suppress aggregation were researched. Each of peptide samples, i.e., a sample prepared by purifying with SP-sepharose BigBeads chromatography for [RHHGP[G]] "RHHGP(G)", a sample prepared by amidating with purified [RHHGP[G]] "RHHGP(G)" for "RHHGP-1," a sample prepared by cleaving [RHHGP[G]] "RHHGP(G)" with Kex2 protease for GLP-1(7-37), and a sample prepared by cleaving RHHGP-1 with Kex2 protease for GLP-1(7-36)NH<sub>2</sub>, was purified to a purity of 98% by eluting with an acetonitrile concentration gradient using the Poros R2 column, and then lyophilized to prepare each sample.

Since the aggregating property is considered to be associated with the solubility of each peptide, the pH dependency of the solubility of each peptide was investigated. The results are shown in Figure 22 and 23. It can be seen that GLP-1(7-37) is low in solubility in the range of pH 5.5 to pH 6.5, i.e., from the weak acid to the neutral condition which is optimum pH for the amidation enzyme reaction condition, indicating that it is inappropriate as a production intermediate.

On the other hand, although [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" start to show decreased solubility at about pH 6.2, they retain adequate solubility at least up to pH 5 or pH 6, and it was confirmed that they could be adequately efficiently reacted even under the amidation enzyme reaction condition.

#### Example 11. Screening of substances having aggregation suppression

In the establishment of the production method of GLP-1(7-36)NH<sub>2</sub> on an industrial level using the production method of the present invention that employs a helper peptide, a substance that increases

the solubility of [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" in the neutral to the weak alkali region and that maintain the solubility of GLP-1(7-36)NH<sub>2</sub> in the weak acid to the weak alkali region, if present, would be more preferred (GLP-1(7-36)NH<sub>2</sub> lags behind GLP-1(7-37) in the study shown in Figure 22, but it was found that it forms precipitates or microcrystals with time in the broad range of pH 5.3 to pH 8.0).

As the substances that are added to the solution so as to be able to maintain the solubility of each peptide, possible candidates include surfactants, sugars, salts, organic solvents and the like. Therefore, Tween 80 and Triton X100 as a surfactant, glucose, mannitol, and sucrose as a sugar, NaCl as a salt, and ethanol, glycerol, and DMSO as an organic solvent were tested for their ability of suppressing aggregation.

Ten mg/ml aqueous solutions of [RHHGP[G]] "RHHGP(G)", "RHHGP-1," and GLP-1(7-36)NH<sub>2</sub> were prepared. To plastic tubes containing 0.1 ml of 300 mM BR buffer, pH 7.9, and 0.1 ml of 10-fold concentrated test sample solutions, 0.8 ml of each peptide solution was added, and pH was adjusted to levels that can easily cause aggregation, such as pH 7.5 to 8.5 for [RHHGP[G]] "RHHGP(G)", pH 8.0 for "RHHGP-1," and pH 6.5 for GLP-1(7-36)NH<sub>2</sub>, and then turbidity (absorbance at 660 nm) with time was monitored. Among the results obtained, the suppressive effects on aggregation by Tween 80, NaCl, and temperature are shown in Figure 23 and Figure 24.

It was found that precipitate formation was strongly suppressed with 0.1% Tween 80 in [RHHGP[G]] "RHHGP(G)" and "RHHGP-1" (Figure 23A), but in GLP-1(7-36)NH<sub>2</sub> aggregation was suppressed by Tween 80 at 0.3% or higher (Figure 23B), NaCl at 100 mM or higher



(Figure 24C), and/or at low temperature (Figure 24D).

From the above results, it was demonstrated that with regard to the pH condition in the actual production system, the addition of a salt and a  
5 surfactant are useful for suppressing the aggregation of the peptide of interest when the method for producing peptides of the present invention is used, and was indicated that the peptide of interest can be produced at high purity and high yields.

10        Example 12. Methods of cleaving in the cleavage site region

Methods of cleaving used when cleaving is carried out at cleavage site regions were investigated. Thus, when the cleavage method for each of the case (1)  
15 cleavage site region 1: cyanization / alkalization, cleavage site region 2: Kex2 protease; (2) cleavage site region 1: Kex2 protease, cleavage site region 2: Kex2 protease; and (3) cleavage site region 1: DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) addition / Kex2  
20 protease, cleavage site region 2: reduction / Kex2 protease was investigated using the peptide of interest as GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub>, it was found that cleavage by a multi-step cleavage method can be carried out by a chemical or enzymatic  
25 treatment when a fusion protein comprising a protective peptide is used in the production method of the present invention.

Results for each method are shown below. In order to enhance hydrophilicity of the polypeptide comprising a helper peptide and GLP-1(7-37) formed  
30 after cleavage and thereby to improve the solubility at neutral pH, 4 contiguous histidine sequences were introduced in all helper peptides.

A. A method of producing GLP-1(7-37) through  
specific cleavage at the cysteine residue  
using method (1)

This method chemically cleaves a cysteine residue  
5 in one cleavage site region (cleavage site region 1)  
when the peptide of interest does not contain  
cysteine. For example, it was confirmed that after  
the fusion protein was expressed and obtained and a  
cysteine residue was cyanized with CADP (1-cyano-4-  
10 dimethylamino-pyridinium tetrafluoroborate), it could  
be specifically cleaved at the N-terminal side of  
cysteine of the cleavage site region by an alkali  
(NaOH) treatment.

Thus, PGGRPSRHKR (SEQ ID NO: 6) was selected as  
15 an amino acid sequence in cleavage site region 1  
adjacent to the amino acid sequence of GCHHHH (SEQ ID  
NO: 5) as a helper peptide, and was introduced into a  
fusion protein. Said fusion protein was dissolved at  
30 mg/ml in 50 mM Tris-HCl, pH 8.2, 5 M urea, and 10  
20 mM DTT (dithiothreitol), and incubated in a 30°C  
incubator for 30 minutes to completely reduce  
cysteine. This was subjected to a gel-filtration  
column (manufactured by Pharmacia, PD10 column) that  
had been equilibrated with 10 mM Tris-HCl, pH 8.2, and  
25 5 M urea to remove DTT.

In order to cyanize cysteine, acetic acid was  
added to a final concentration of 0.1 M, and CADP at  
4-fold mole greater amounts than cysteine was added  
thereto and was reacted at 30°C for 1 hour.  
30 Cyanization reaction was confirmed by determining the  
residual SH groups with DTNB.

The above fusion protein was specifically cleaved  
at the cyanized cysteine site by removing an excess of  
reagents with a PD10 column equilibrated with 10 mM  
35 acetic acid and 5 M urea, adding NaOH to a final

concentration of 50 mM, and then leaving it at room temperature for 30 minutes. The rate of cleavage was about 50 to 70%.

5 The other cleavage site region (cleavage site region 2) was subjected to a cleavage treatment in a similar manner to Example 7.

B. Cleavage of a cleavage site region with Kex2 protease using method (2)

10 It was designed that while each cleavage site region has a Kex2 protease cleavage site region, the amino acid sequence of one cleavage site region (cleavage site region 2) is hardly cleaved under the reaction condition for the cleavage of the other cleavage site region (cleavage site region 1).

15 It is known that in the optimization of a cleavage recognition sequence in the cleavage site region of Kex2 protease, the charge of the amino acid residue at the P4 subsite, in addition to the P1 and P2 subsites, greatly influences the activity of the enzyme and that the fusion protein is not cleaved at all under a certain condition especially when an acidic amino acid is present at the P4 subsite (Japanese Unexamined Patent Publication (Kokai) 9(1997)-296000). By utilizing this, for example, by  
20 introducing aspartic acid (D) into the P4 subsite as the amino acid sequence SDHKR (SEQ ID NO: 8) of cleavage site region 2 adjacent to an amino acid sequence comprising HRHKRSHHHH (SEQ ID NO: 7) as a helper peptide, 90% or greater was cleaved with Kex2  
25 protease in the cleavage of cleavage site region 1 in the fusion protein but cleavage site region 2 was protected from the cleavage. The result revealed that cleavage site region 1 can be specifically cleaved by  
30 introducing aspartic acid into the P4 subsite.

35 In an ion exchange chromatography and a column

chromatography (for example, Cellulofine C-200 column chromatography) having a gel filtration ability that were carried out in order to isolate the obtained GLP-1(7-37) having a helper peptide added thereto, the  
5 desired GLP-1(7-37) having a helper peptide added thereto was specifically adsorbed. Some of the unreacted fusion protein also adsorbed but they were readily separated by a salt concentration gradient. The recovery was 94%. The chromatography can also be  
10 applied to GLP-1(7-36)NH<sub>2</sub> having another helper peptide added thereto.

Subsequently, after an amidation modification reaction, Kex2 protease cleavage treatment was carried out at cleavage site region 2 in order to excise GLP-1(7-36)NH<sub>2</sub> from GLP-1(7-36)NH<sub>2</sub> having a helper peptide  
15 added thereto. It was found that under the first cleavage condition which contained urea (denaturant) and which was alkaline, Kex2 protease did not cleave cleavage site region 2, but in the absence of urea  
20 cleavage site region 2 could be recognized by choosing an appropriate pH. It was shown that the optimum pH was 6.5 to 7.3 and thus cleavage was not easy at pH 8.2 which is a cleavage reaction condition in cleavage site region 1.

This is because the above GLP-1(7-36)NH<sub>2</sub> having a helper peptide added thereto is different from the fusion protein having a protective peptide further added thereto and is soluble in a wide pH range even in the absence of urea. Thus, Kex2 protease was  
25 allowed to react and the peptide was separated and determined with a reverse phase HPLC with the cleavage of 98% or greater being defined as complete. Since the system does not contain urea, the activity loss of Kex2 protease hardly occurs. Thus, it has an  
30 advantage that the amount of Kex2 protease required is  
35

extremely small, with a molar ratio to the substrate being 1:20000 to 1:40000.

As mentioned above, it was demonstrated that GLP-1(7-37) and GLP-1(7-36)NH<sub>2</sub> could be produced by using the same enzyme in cleavage treatment of each cleavage site region.

C. Cleavage method utilizing a specific modification of cysteine using method (3)

This method is almost similar to method (2) and, when the peptide of interest does not contain cysteine residues, it modifies the cysteine residue introduced into the P4 subsite of one cleavage site region (cleavage site region 2), i.e. it protects the cleavage site region 2 from cleavage at the time of Kex2 protease cleavage reaction at the other cleavage site region (cleavage site region 1) by treating the side chain of cysteine with DTNB (dithionitrobenzoic acid), carries out reduction after a peptide comprising GLP-1(7-37) having a helper peptide added thereto was obtained, to thereby cleave cleavage site region 2 with Kex2 protease. Representative examples of such modification reactions include sulfonation and asymmetric disulfidization with DTNB, but are not limited to these reactions as long as the method gives a negative charge to the cysteine side chain. For example, the amino acid sequence of cleavage site region 2 adjacent to the amino acid sequence comprising HRHKRSHHHH (SEQ ID NO: 7) as a helper peptide is introduced into a fusion protein as SCHKR (SEQ ID NO: 24).

The fusion protein designed as above was expressed, the fusion protein obtained was subjected to a DTNB treatment, and cysteine was made an asymmetric disulfide. After confirming the complete modification of cysteine, cleavage treatment reaction

with Kex2 protease was carried out, and then it was confirmed that a peptide comprising GLP-1(7-37) having a helper peptide added thereto in an quantitative manner could be obtained. Thus, by changing the P4  
5 subsite of the amino acid sequence recognition site of Kex2 protease at cleavage site region 2 to cysteine, and by introducing a negative charge in a side chain-specific manner, cleavage site region 2 was protected from cleavage at the time of cleavage with Kex2  
10 protease at cleavage site region 1.

Then, purification and an amidation reaction was carried out as in the above B, DTT was added to reduce GLP-1(7-36)NH<sub>2</sub> having a helper peptide added thereto, hydrophobic column chromatography was carried out to  
15 purify to a purity of 98%, and then the product was lyophilized. When the product was dissolved at 5 mg/ml in 20 mM BisTris, pH 7.0, and 2 mM calcium chloride, 1000 units/ml of Kex2 protease was added thereto, and the mixture was reacted at 30°C, GLP-1(7-  
20 36)NH<sub>2</sub> having a helper peptide added thereto was specifically cleaved to give GLP-1(7-36)NH<sub>2</sub>.

Although cleavage site region 2 was in a reduced state in this example, it is possible, needless to say, to modify it to have a positive charge and  
25 thereby to change its reactivity to Kex2 protease.

#### Industrial Applicability

In accordance with the present invention, a method for producing biologically active peptides efficiently and at low cost on an industrial scale is  
30 provided. Specifically, it was demonstrated, as described in Examples of the present invention, that GLP-1 derivatives for which industrial production has been difficult can be produced at high purity and high yields. The production method of the present  
35 invention can be applied to the production of

biologically active peptides other than GLP-1 derivatives and thus are industrially extremely useful.

## ABSTRACT

[In the production] Production of peptides of  
interest having [a] desired biological activity ~~using~~  
5 ~~genetic engineering technology, it is characterized~~  
~~that~~ whereby said peptide of interest is expressed  
with the addition of a helper peptide to said peptide  
of interest. This method of production results in  
[the] enhancement in the purification efficiency and  
10 [the] yield of the peptide of interest through the  
prevention of aggregation during the recovery and/or  
purification process by adjusting isoelectric points,  
[the] enhancement of recovery in ion exchange  
chromatography, [the] enhancement of solubility of a  
15 fusion protein during the enzyme reaction for release  
the peptide of interest by enzymatic cleavage when the  
peptide of interest is expressed as a fusion protein  
with another peptide, [the] enhancement of solubility  
of said peptide during the enzyme reaction when the  
20 enzymatic modification of the peptide of interest is  
required, and the like.